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# The green yeast uses its plant-like clock to regulate its animal-like tail

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*Chlamydomonas reinhardtii* is a study in contrasts: It is a soil-dwelling, unicellular algae, but it can swim; it practices photosynthesis like a plant, but has many distinctly animal-like genes; it is called the green yeast—referring to its shape and size—yet, genetically, it bears less resemblance to the fungi than to plants and animals; and finally, although it harvests light via chloroplasts for energy as many plants do, *Chlamydomonas* possesses a structure that strangely resembles the eye of an animal that, in combination with its flagella, permits orientation within the soil. So, *Chlamydomonas* is a plant-animal, still related to the last common ancestor of the two kingdoms.

The green yeast has been a denizen of the laboratory for decades. It is easy to grow in liquid cultures and has fascinating morphology and behaviors. Relatively recently, *Chlamydomonas* research has touched on applied topics that would use metabolism for the purposes of industrial production, as well as basic research. A major topic concerns the eyespot, which is recognized microscopically by carotenoid-rich lipid droplets (Nagel et al. 2003). These spots catch light and reflect it back toward membrane-bound photoreceptors, channel rhodopsin, in an apparent efficiency exercise. Biophysicists have been using the *Chlamydomonas* eyespot to study photoreception, leading to the expression of channel rhodopsin in a number of cell types that are not typically photoreceptive.

As of last year, *C. reinhardtii* has joined the post-genomic era, with a genome annotation confirming its evolutionary history and suggesting a mechanistic basis for its ability to persist in diverse conditions (Merchant et al. 2007). The genome encodes >15,000 genes. *Chlamydomonas* has a large number of transporters that likely support growth in many different situations, even without light and using added carbon sources. It has a plethora of duplicated genes, and those genes have introns that commonly have microsatellite-like repeats, making *Chlamydomonas* an interesting organism with

which to study basic molecular genetic mechanisms. Together with the possibility of practicing forward and reverse genetics, the genome sequence makes it possible to use *Chlamydomonas* to unravel many basic biological processes. A study in this issue of *Genes & Development* moves the circadian clock to the head of that list (Matsuo et al. 2008).

## Daily biological timing

Circadian clocks provide a temporal structure for organismal biology that matches the daily cycle in the environment (Merrow et al. 2005). The rotation of the earth about its axis creates a periodic supply of a basic energy source for photosynthesizers, which are upstream of all other life on earth. In addition, cellular metabolic processes should be modulated in their reaction kinetics due to the accompanying 24-h temperature cycles. The circadian system, found in organisms from all phyla, is widely viewed to be an evolutionary adaptation to the regular, predictable changes in the environment according to time of day and time of year. Although, in nature, circadian clocks are always synchronized to the daily 24-h light–dark cycles (or downstream—e.g., temperature—cycles that are dependent on them), when some organisms are released to constant conditions, a self-sustained oscillation, called a circadian rhythm, persists, sometimes for years. That the rhythms are endogenous points to their genetic basis.

Entrained circadian oscillations, as well as free-running circadian rhythms, regulate biology from the level of gene expression to behavior. Without a circadian system, organisms survive less well or may become seriously ill, shown respectively in unicells and mice (Yan et al. 1998; Fu et al. 2002; Turek et al. 2005). Among our own species, it is the circadian clock that dictates if we are larks or owls (early or late to sleep and arise) (Xu et al. 2005). It does this because the genetically complex clock of different individuals can respond differently to entraining stimuli, resulting in early or late phasing through the day (Roenneberg et al. 2007). An understanding of clock mechanisms is thus of interest to all biologists, as the clock regulates most of biology.

The circadian clock is said to be cell-based. The intact

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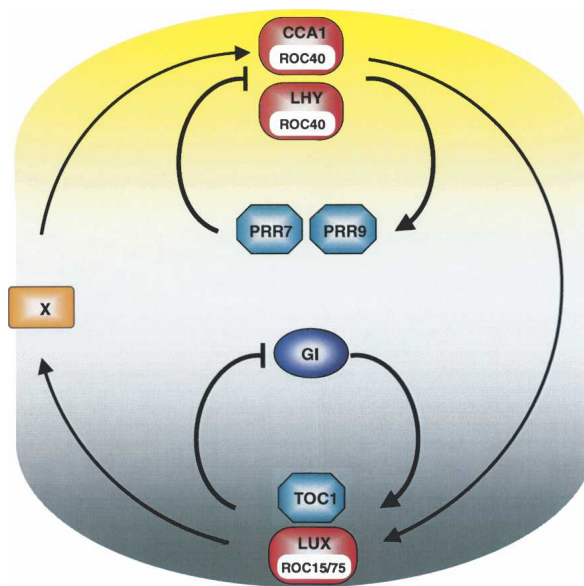
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rat shows a free-running rhythm, but so do its organs, as well as its fibroblast cells that have been kept in tissue culture for decades (Balsalobre et al. 1998; Yamazaki et al. 2000). Through mutant screens, the molecular regulators of cell-based oscillations have been characterized in prokaryotes (*Synechococcus elongatus*), fungi (*Neurospora crassa*), plants (*Arabidopsis thaliana*), and animals (*Drosophila melanogaster* and *Mus musculus*). So-called clock genes use a similar regulatory formula in each of the model systems (e.g., see Fig. 1); namely, a highly complex, autoregulatory transcription-translation feedback loop (Young and Kay 2001; Brunner and Schafmeier 2006; Ko and Takahashi 2006; Yakir et al. 2007). Yet, a phylogenetic comparison of these components suggests that circadian clocks have evolved independently in animals, plants, fungi, and the cyanobacteria.

The feedback loop construction in the model organisms is typically characterized through genetic interactions, but is supported in numerous cases with molecular or biochemical data (e.g., protein-protein interaction, promoter binding, etc.). The building blocks include transcriptional activator complexes that regulate expression of regulators that feed back negatively on the activator. In animals, the activator complex is composed of two PAS domain proteins with basic helix-loop-helix (bHLH) DNA-binding domains. In *Neurospora*, the corresponding heterodimer components also bear PAS domains, and in addition have GATA-type Zn-finger DNA-binding domains. In plants, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), two partially redundant single MYB DNA-binding domain proteins serve as the activators for the feedback loop. The balance between half-life, cellular localization, and post-translational modification of the clock proteins are all critical factors in circadian timing. The central transcriptional loop, in addition to being self-regulating, also controls a large number of other genes, resulting in diverse clock-controlled processes in the cell.

This is a seemingly neat formula, but it is unfortunately an oversimplification, which is where the advantage of an additional and simple model organism like *Chlamydomonas* comes in. In established model systems, the central loop is much larger than what was originally conceived. There are interlocked loops, interdependent loops, and even self-sustained rhythms or circadian entrainment in the absence of critical clock genes (Merrow et al. 1999; Steinlechner et al. 2002; Roenneberg et al. 2005). These observations do not diminish the importance of the clock genes (they are so defined due to their dominant control over properties that are shared with all circadian systems, such as self-sustained rhythms or phase of entrainment), yet they indicate that there is more to the clock mechanism than forward genetics and the feedback loop model have revealed. Perhaps the question should be: At what locus in the clock mechanism do the transcriptional feedback loops operate, and what other types of mechanisms in the cellular clock are at work?



**Figure 1.** Potential similarity of the circadian clocks of *Arabidopsis* and *Chlamydomonas*. The core transcriptional feedback loops of the circadian clock of *Arabidopsis* is schematically outlined. The *Chlamydomonas* clock genes *ROC40*, *ROC15*, and *ROC75* encode putative transcription factors related to components of the feedback loops of the circadian clock of *Arabidopsis* (white text fields). *ROC40* encodes, like the two partially redundant *Arabidopsis* transcription factors CCA1 and LHY, a single MYB domain protein. *ROC15* and *ROC75* encode GARP (a subclass of MYB) domain transcription factors like LUX/PCL1. CCA1 and LHY oscillate with a peak in the morning. LUX/PCL1, together with TOC1, a PRR, oscillates ~180° out of phase with a peak in the evening. CCA1 and LHY activate expression of PPR7 and PPR9, which in turn inhibit expression of CCA1 and LHY, constituting the so-called morning oscillator. The evening oscillator is composed of LUX and TOC1, which negatively regulate GI. The clock protein GI indirectly supports accumulation of TOC1 in the dark. In the light, GI stabilizes by complex formation the blue-light-sensing LOV-KELCH-F-box protein ZTL, which targets TOC to degradation via the ubiquitin/proteasome pathway. GI and ZTL do not interact in the dark, and both proteins are rapidly turned over, leading to reduced turnover and thus sharp accumulation of TOC after light-to-dark transition. Morning and evening oscillators are coupled. The evening oscillator supports, via an unidentified component, X accumulation of CCA1 and LHY, which in turn negatively regulate TOC and LUX. This system of interconnected loops is supported by a number of components that are crucial for robust oscillation in constant or entrained condition. Many clock and clock-associated components are phosphorylated by kinases and are turned over by the proteasome via light-regulated F-box proteins of the ZTL family.

The answer could relate to the kinases and phosphatases that are associated with all clock systems. Indeed, in eukaryotes, these are much older genes than are the clock genes themselves, and the clock genes do not function without them—leaving the possibility that the clock gene networks may have been recruited by a primordial clock that is based on (oscillatory) metabolic processes such as phosphorylation. The most intriguing

data concerning phosphorylation as a clock mechanism comes from the cyanobacteria clock, which has been reconstituted in a test tube with just three proteins and ATP (Nakajima et al. 2005). KaiC autophosphorylates and is positively and negatively regulated in this function by KaiA and KaiB proteins, respectively. The period of a full cycle of phosphorylation and dephosphorylation of KaiC is ~24 h, and the clock-in-a-tube—a phoscillator—shows other clock properties (e.g., temperature compensation) that were not necessarily predicted to derive from enzyme kinetics. The eukaryotic clock mechanism may be a compilation of primitive regulatory mechanisms, such as a phoscillator (Morrow et al. 2006), as it interacts with specialized hands of the clock, such as species-specific or organ-specific transcriptional feedback loops.

### The molecular mechanism of the *Chlamydomonas* clock

The circadian clock of *Chlamydomonas* was described almost 40 years ago (Bruce 1970). The cells are phototactic in liquid culture, moving themselves into an optimal position to harvest photons. Consequently, there is a circadian rhythm in growth rate, perhaps similar to the gating of cell division that is observed in many cells in mammalian tissues to distinct windows during the day. Although pulses of UV light exposure will kill *Chlamydomonas* cells, they are more susceptible to it at the beginning of the night and least susceptible in the morning hours, revealing a circadian rhythm in an important ecological adaptation (Nikaido and Johnson 2000). Mutants that are defective in circadian rhythms were isolated (Bruce 1970). These mutations mapped to the nuclear and chloroplast genome, but they generally correspond to clock-controlled genes. To date, no clock genes have been identified to suggest the identity of a feedback loop regulating transcription (Mittag et al. 2005). Furthermore, an elegantly elaborated clock output mechanism in the green yeast concerns translational regulation involving RNA-binding proteins and 3' untranslated regions (UTRs) of mRNAs (Mittag 2003). Thus, the nature of the circadian clock of *Chlamydomonas*, in particular, and algae, in general, remained obscure.

To address this question, Ishiura and colleagues (Matsuo et al. 2006) adapted some tricks that were used to crack the cyanobacteria clock; namely, perfecting a high-throughput screen involving luciferase, real-time monitoring, and a clock-regulated promoter. They fused a codon-optimized firefly luciferase gene (*C. reinhardtii* is GC-rich) to the psbD promoter and integrated the chimeric gene into the chloroplast genome. In their latest work (Matsuo et al. 2008), the team used the psbD-luc reporter strain to conduct a forward genetic screen for clock genes of *Chlamydomonas*. Interestingly, the original reporter strain had a low-amplitude rhythm in luciferase expression, but by crossing into different wild-type strains, a distribution of amplitudes was revealed, and a

stable, high-amplitude oscillation was captured in one of these. This experiment shows that multiple traits (genes) contribute to the robustness of the circadian clock and/or its outputs. The circadian clock has other properties, and the progeny of the crosses told a different story concerning these. For circadian period, the distribution was constrained, as expected, to close to 24 h, with no notable changes in the F1s except for a slightly wider distribution of periods. The phase on the first day following release into constant conditions (this is not the same as entrained phase) showed an intriguing bimodality, with half of the phases in the subjective morning, and half in the subjective evening. This suggests that the original host strain is half a day out of phase with the three other wild-type strains that were used, and that there are relatively few genes that control phase, or that they come in a form that confers a dominant or semi-dominant phenotype. The cross data in general could be exploited statistically to better understand the basis of genetic control over clock properties, and their relationship to one another. For instance, like physical oscillators, a relationship between period and phase is common. This relationship appears to be violated here, and it undoubtedly tells us something about the clock mechanism.

From screening 96 progeny from each cross, an isolate with a robust and high-amplitude oscillation was selected and backcrossed to generate a strain that was suitable for a high-throughput mutant screen. The reporter strain was transformed with a hygromycin resistance marker cassette and ~16,000 transformants were screened for defects in the circadian bioluminescence rhythm. Matsuo et al. (2008) identified 105 clones that displayed an altered luminescence rhythm of chloroplasts (*roc*). Fifty of these mutants were tagged with a single hygromycin cassette and the *roc* phenotype segregated with the inserted hygromycin marker. Finally, 32 different *roc* mutants were classified according to altered period length, phase, and amplitude. Most mutants (78%) displayed a (persistent or dampening) low-amplitude rhythm or were arrhythmic. The observed defect of the luminescence rhythm was paralleled by a corresponding defect in the circadian growth rhythm (with exception of *roc81*; see below), indicating that general clock functions rather than specific output pathways were affected.

The loci targeted by the hygromycin cassette were determined for 32 mutants. Seven genes were independently targeted twice, suggesting an insertional bias of the hygromycin cassette. Based on sequence analysis, the mutated *roc* genes have roles in flagellar functions (six genes), membrane trafficking (four genes), apoptosis-induced cytoplasmic vacuolization (one gene, *Alix*) (Chattellard-Causse et al. 2002), transcriptional regulation and RNA metabolism (nine genes), and ubiquitin-dependent protein turnover (two genes). Furthermore, three kinase-encoding genes involved in signaling, DNA damage response, and gene silencing (MAPKKK, ATR1, and MUT-9) were identified.

In terms of what these mutants reveal concerning the

clock mechanism, flagellar functions seem to be required for the amplitude of the chloroplast rhythm, since all mutants in this group displayed low-amplitude rhythms. The low-amplitude rhythm is observed in constant darkness and thus is not due to impaired phototaxis. The mutant phenotype could be due to loss of motility per se, or to a reduced metabolism of strains that are lacking a functional flagellum and thus have a lower demand for ATP.

The description of membrane-trafficking components as regulating the clock is novel, and a putative clock-related function of membrane trafficking is not immediately obvious. One gene of this group, *ROC81*, encodes a protein with similarity to the N-terminal region of VTC4, a *trans*-membrane subunit of the yeast vacuolar transporter chaperone involved in regulation of microautophagy, vacuolar phosphate accumulation, and H<sup>+</sup>ATPase activity (Uttenweiler et al. 2007). The *roc81* mutant was the only one that specifically affected the rhythm of chloroplast bioluminescence while the circadian growth rhythm was undisturbed. Thus, *ROC81* most likely has a function in a specific output pathway of the clock involved in signaling between cytosol and chloroplasts.

The largest group of *ROC* genes encodes transcriptional regulators. *ROC40* contains a single MYB DNA-binding domain and *ROC15/75* contains a GARP domain (a subclass of MYB), similar to the *Arabidopsis* circadian transcription factors CCA1 and LHY and to LUX ARRYTHMO/PHYTOCLOCK 1 (LUX/PCL1), respectively. This suggests that the two types of transcription factors are conserved in the circadian systems of the green lineage (Fig. 1). *ROC66* contains a B-box zinc-finger domain and a CONSTANS/CONSTANS-LIKE/TOC (CCT) motif. Both domains are present in CONSTANS (CO), which mediates between the circadian clock and the control of flowering in *Arabidopsis* (Suarez-Lopez et al. 2001). *ROC56* and *ROC76* have no similarity to known clock components of *Arabidopsis*. However, they contain zinc-finger and basic leucine-zipper DNA-binding motifs and thus are good candidates for transcription factors that may form interlocked feedback loops of a *Chlamydomonas* circadian clock.

*XRN1* (*ROC86*) encodes a 5′–3′ RNA exonuclease, suggesting circadian regulation on the level of RNA turnover. This complements evidence indicating that the RNA-binding protein CHLAMY1 regulates the circadian phasing of an abundance of RNA transcripts by interacting with UG repeats in the 3′UTR (Mittag 2003; Iliev et al. 2006). A putative binding site of CHLAMY1 is present in the 3′UTR of the *ROC40* transcript. The system designed by Ishiura (Matsuo et al. 2008) can thus reveal post-transcriptional regulatory mechanisms, as has long been appreciated in the circadian clock of *Chlamydomonas* and also of *Gonyaulax polyedra*, a marine diatom (Mittag et al. 1994).

*SKP1* (*ROC80*) encodes S-phase kinase-associated protein 1—a subunit of the Skp1/cullin/F-box (SCF) E3 ubiquitin ligase complex—and *ROC114* encodes an F-box-containing protein. This suggests that regulated protein

turnover is important for the circadian clock of *Chlamydomonas*. Components of the ubiquitin–proteasome pathway, in particular F-box proteins, are crucial for the circadian clocks in all eukaryotic model systems. All known F-box proteins associated with the circadian clock mechanism seem to regulate turnover of components of the autoregulatory transcriptional feedback loops (Somers et al. 2000; Grima et al. 2002; He et al. 2003; Shirogane et al. 2005; Busino et al. 2007; Godinho et al. 2007; Siepka et al. 2007). It will be interesting to learn whether *ROC114* targets one of the *ROC* transcription factors to proteasomal degradation. It should be pointed out that *ROC114* neither encodes Kelch repeats nor a blue-light-sensing LOV domain, both present in the *Arabidopsis* F-box protein ZTL that mediates light-dependent degradation of TIMING OF CAB EXPRESSION 1 (TOC1) (Somers et al. 2000), a core component of the clock.

Turnover and function of clock proteins is regulated by post-translational modification; in particular, by phosphorylation (Brunner and Schafmeier 2006; Gallego and Virshup 2007). The kinases and phosphatases involved in this regulation are often essential for other cellular processes and may not be identified readily by an insertional screen for clock mutants. *ROC78* encodes a nonessential MAPKKKK. MAPK pathways have been implicated in signaling to the clock in mammals, flies, and fungi (Coogan and Piggins 2004; Weber et al. 2006; Vitalini et al. 2007). ATR1 (*ROC69*) is related to an ATR/RAD3 DNA damage-sensing protein kinase and MUT-9 (*ROC94*) is a kinase involved in gene-silencing and DNA damage sensitivity. A connection between DNA damage, cell cycle, and the circadian clock has also been established in other species (Gery et al. 2006; Pogueiro et al. 2006), suggesting that these kinases have corresponding functions in evolutionarily diverse circadian systems.

It should be noted that no *ROC* gene has been found with extended similarity to TIMING OF CAB EXPRESSION 1 (TOC1), a pseudo-response regulator (PRR) homolog in the core of the *Arabidopsis* feedback loops (Strayer et al. 2000), or to its paralogs PRR9, PRR7, and PRR5, which regulate flowering time through a photoperiodic pathway dependent on CONSTANS (Nakamichi et al. 2007). Furthermore, no *ROC* components with similarity to GIGANTEA (GI) and the blue-light-dependent F-box protein ZEITLUPE (ZTL), which together regulate the stability of TOC1 (Kim et al. 2007), were identified by the screen. Since obvious homologs of TOC1, GI, ZTL, and ZTL-like proteins are not found in the genome of *Chlamydomonas*, the system of transcriptional feedback loops of the circadian clock in *Chlamydomonas* might be less complex than in higher plants.

Functionally redundant genes and genes with essential functions in other cellular processes, by their nature, will not be represented in the collection of *roc* mutants. Furthermore, components of specific input and output pathways may not be represented. Although the *Chlamydomonas* genome contains a gene with similarity to EARLY FLOWERING 4 (ELF4), involved in regulation of

light input into the *Arabidopsis* clock, as well as a number of putative red-light (*phytochrome*) and blue-light (*cryptochrome*) photoreceptor genes (Mittag et al. 2005), these were not identified by the screen. They may yet have a function in the *Chlamydomonas* clock.

In summary, the transcriptional feedback loops of the *Chlamydomonas* clock features a transcriptional feedback loop that is most similar to those of the higher plant clock (Fig. 1). Notably, there is an absence of genes in the genome with significant similarity to the mammalian clock genes *Clock*, *Bmal1*, *Period*, *Rev-erba*, and *RorA*; to the *Drosophila* clock genes *clock*, *period*, *timeless*, *vrille*, and *pdp1*; and to the *Neurospora* clock genes *frequency*, *white collar 1*, and *white collar 2*. However, it should be underscored that the identified *Chlamydomonas* ROC components classified as transcriptional regulators share functionally important domains with *Arabidopsis* clock proteins but are not true homologs.

### The *Chlamydomonas* clock in context

The Ishiura laboratory (Matsuo et al. 2006, 2008) has identified and partially characterized many clock genes of *Chlamydomonas*. Their data suggest that transcriptional feedback loops are part of the circadian system of *Chlamydomonas*. The components making up these putative loops share characteristic domains of transcription factors of the circadian clock of *Arabidopsis*. These results, together with in silico interrogation of the *Chlamydomonas* genome for clock gene homologs, suggest that transcriptional feedback loops of the *Chlamydomonas* clock are similar to those of *Arabidopsis*. *Chlamydomonas* may therefore be an appropriate system to learn about minimal (common) transcriptional circuitry of circadian clocks of the green lineage.

The data of Matsuo et al. (2006, 2008) confirm that the *Chlamydomonas* clock relies on post-transcriptional regulation on the level of RNA metabolism, protein phosphorylation, and turnover (Schmidt et al. 2006; Wagner et al. 2006)—functions that are common hallmarks of eukaryotic clocks. Biochemical and cell biological tools such as specific antibodies and GFP fusion proteins will be required for the next level of analysis of the circadian system of *Chlamydomonas*.

Surprisingly, the genetic screen of Matsuo et al. (2008) identified a large number of candidate clock genes with no obvious direct connection to the proposed transcriptional feedback loops or known clock mechanisms. These ROC genes can be classified into flagellar genes and genes regulating membrane trafficking, in particular, of vacuolar membranes. Since the flagellum and the vacuolar ATPase consume large amounts of energy, mutations in these functions could affect the clock via metabolic pathways. Given that genes of these classes have not been found previously to be part of the circadian system in other organisms, the *Chlamydomonas* system may provide a unique tool to learn about their impact on the circadian system.

Finally, we recall the puzzling example of *Acetabularia*,

the giant unicellular algae that shows a self-sustained circadian rhythm even if the nucleus has been “surgically” removed (Schweiger et al. 1964). The dissociation of *Chlamydomonas* nuclear and chloroplast rhythms shown in the work here suggests that the circadian system of this microscopic algae is constructed similarly to *Acetabularia*. This phenomenon might also be related to the striking demonstration of two oscillators in the single-celled *Gonyaulax polyedra* (Roenneberg and Morse 1993). Thus, this simple cell might be an apt tool for investigations into how a pacemaker (read, nucleus) interacts with peripheral oscillators (read, chloroplasts), and vice versa, and how a circadian system adjusts to the seasons. In this case, the complex, multicomponent circadian system is entirely in a single cell that is a cousin of both plants and animals.

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